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Cooperative and Antagonistic Interplay between PU.1 and GATA-2 in the Specification of Myeloid Cell Fates

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Summary

PU.1 and GATA transcription factors appear to antagonize each other's function in the development of distinct lineages of the hematopoietic system. In contrast, we demonstrate that PU.1, like GATA-2, is essential for the generation of mast cells. *PU.1*−/− hematopoietic progenitors can be propagated in IL-3 and differentiate into mast cells or macrophages upon restoration of PU.1 activity. Using these progenitors and a conditionally activatable PU.1 protein, we show that PU.1 can negatively regulate expression of the *GATA-2* gene. In the absence of GATA-2, PU.1 promotes macrophage but not mast cell differentiation. Reexpression of GATA-2 in such progenitors enables the generation of mast cells. We propose a developmental model in which cooperative function or antagonistic crossregulation by PU.1 of *GATA-2* promotes distinct myeloid cell fates.

Introduction

The hematopoietic system develops from a self-renewing stem cell (HSC) that generates a hierarchical array of developmental intermediates consisting of multipotent and lineage-committed progenitors which differentiate into erythrocytes, megakaryocytes, and cells of the innate and adaptive immune system. It represents a leading developmental system for analyzing regulatory proteins that specify distinct cell fates. Structurally diverse transcription factors have been shown to play critical roles in lineage specification via both loss-of-function (gene targeting) and gain-of-function (ectopic expression) experiments (Shivdasani and Orkin, 1996; Glimcher and Singh, 1999). The combinatorial mechanisms by which these transcription factors specify distinct cell fates are poorly understood.

The Ets family transcription factor, PU.1, is necessary for the generation of myeloid and lymphoid but not erythroid or megakaryocytic lineages of the hematopoietic system (Scott et al., 1994; McKercher et al., 1996; Singh et al., 1999). The blocks to macrophage and B cell development caused by mutation of the *PU.1* gene are more severe than defects in neutrophil and T cell development (Anderson et al., 1998; DeKoter et al., 1998; Spain et al., 1999). *PU.1*−/− fetal liver hematopoietic progenitors proliferate in response to multilineage cytokines such as IL-3, but are impaired in their responsiveness to the lineage-restricted cytokines GM-CSF, G-CSF, M-CSF, and IL-7 (Scott et al., 1997; DeKoter et al., 1998, 2002). PU.1 appears to directly activate transcription of genes encoding subunits of the receptors for these lineage-restricted cytokines (Singh et al., 1999; DeKoter et al., 2002). In addition to regulating cytokine responsiveness, PU.1 directs the differentiation of hematopoietic progenitors into macrophages, neutrophils, and B lymphocytes (DeKoter et al., 1998; DeKoter and Singh, 2000).

Although PU.1 is expressed in mast cells (Galson et al., 1993), its requirement in mast cell development has not been genetically analyzed. Mast cell progenitors can be enumerated by colony-forming assays using IL-3 and SCF (Lantz and Huff, 1995). Such progenitors, derived from bone marrow or fetal liver, can also be expanded into cell lines using IL-3. Under these culture conditions, the progenitors give rise to immature mast cells representing various stages of development (Galli et al., 1982). These cells contain variable numbers of cytoplasmic granules and express c-kit as well as the high-affinity IgE receptor, FcεRI. Many of these cell lines express transcripts encoding the mast cell proteases mMC-CPA and mMCP-5, suggesting these protease genes are induced early in mast cell development (Reynolds et al., 1989; McNeil et al., 1991). Given that PU.1 does not regulate expression of the IL-3 receptor (DeKoter et al., 1998), we reasoned that it should be possible to analyze the function of PU.1 in mast cell differentiation by expanding mutant hematopoietic progenitors in IL-3.

The transcription factor GATA-2, like PU.1, is essential for the development of multiple hematopoietic lineages but appears to regulate the survival/proliferation of HSC and multipotential progenitors (Tsai et al., 1994). GATA-2 has also been shown to be specifically required for mast cell differentiation by expanding yolk sac progenitors from *GATA-2* null embryos in IL-3 and SCF (Tsai and Orkin, 1997). Under these conditions, wild-type yolk sac cells give rise to both macrophages and mast cells. However, *GATA-2*−/− yolk sac progenitors generate only macrophages. This demonstrates that GATA-2 is required for the generation of mast cells but dispensable for macrophage differentiation. GATA-2 expression is highest in proliferating mast lineage cells and is downregulated in differentiated tissue mast cells (Jippo et al.,

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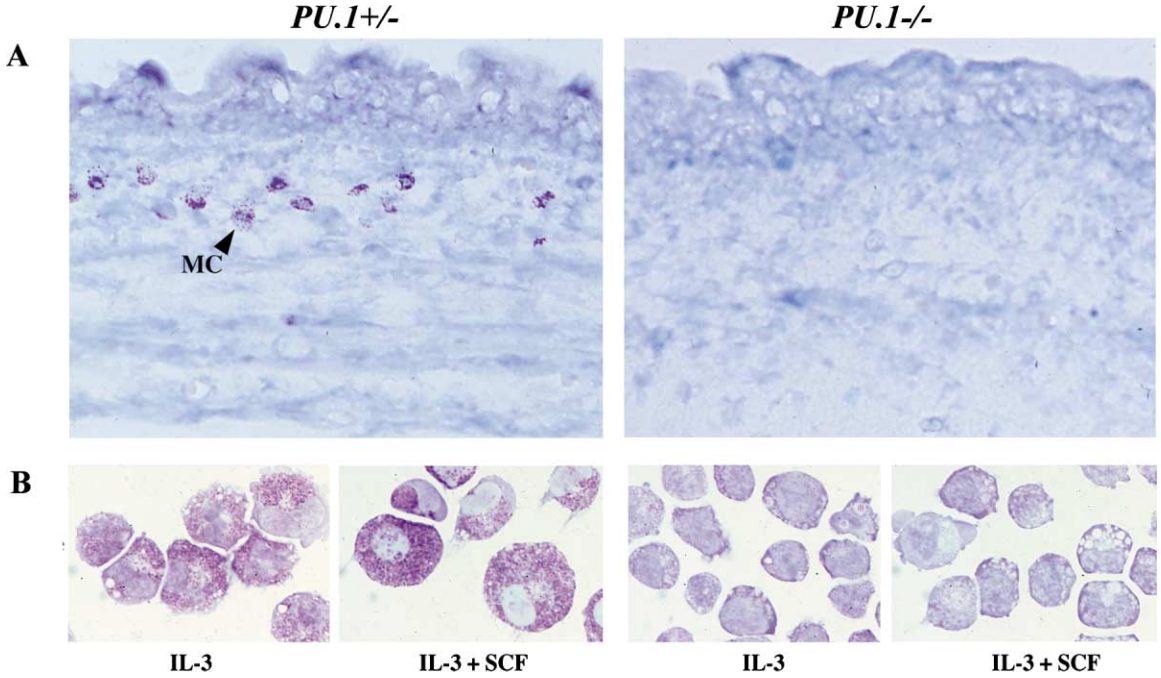

Figure 1. PU.1 Is Required for the Development of Mast Cells

(A) Day 16.5 *PU.1*+/− or −/− embryonic dorsal skin sections were stained with acidified toluidine blue. Mature mast cells (MC) are recognized by metachromatic staining of their granules. *PU.1*+/− embryos contained approximately 400 mast cells/cm of dorsal subcutaneous tissue (left panel) whereas *PU.1*−/− embryos contained no observable mast cells (right panel).

(B) IL-3-dependent cell lines established from day 14.5 *PU.1*+/− (left panels) or −/− (right panels) hematopoietic progenitors. Cells cultured in the presence of either IL-3 or IL-3 and SCF were stained with toluidine blue. Original magnification 1000×.

1996). The latter cells express the related factor GATA-1. Consistent with its pattern of expression, GATA-1 is not required for mast cell development (Pevny et al., 1995). Nevertheless, it appears to regulate the mature phenotype of tissue mast cells (Harigae et al., 1998).

Recently, PU.1 and GATA family transcription factors have been shown to antagonize each other's activities (Rekhtman et al., 1999; Zhang et al., 1999, 2000; Nerlov et al., 2000). PU.1 and GATA-1,2 have been shown to physically interact through their DNA binding domains. Such interaction results in mutual antagonism of their transactivating abilities when they are ectopically expressed in heterologous cell lines. It has been suggested that this direct antagonism between PU.1 and GATA-1 proteins, which results in inhibition of GATA-1 DNA binding activity, could account for the block to erythroid differentiation caused by overexpression of PU.1 (Zhang et al., 2000). Consistent with this explanation, PU.1-mediated inhibition of erythropoiesis in *Xenopus* embryos can be suppressed by overexpression of GATA-1 (Rekhtman et al., 1999). Conversely, ectopic expression of GATA-1 inhibits myeloid differentiation that is dependent on PU.1 (Nerlov et al., 2000). In this case, it has been argued that the interaction of GATA-1 with PU.1 prevents recruitment of coactivators to PU.1-regulated myeloid genes (Zhang et al., 1999). Although GATA-2 has been shown to interact with PU.1 and the two factors can mutually antagonize each other's transactivation functions, the biological relevance of this inhibitory interaction remains unclear. Furthermore, mast lineage cells express both PU.1 and GATA-2, raising the possibility that in this lineage the two factors may function cooperatively rather than antagonistically to specify cell fate.

In this study, we establish a requirement for PU.1 in the development of mast lineage cells. Using *PU.1*−/− hematopoietic progenitor lines, we uncover evidence for cooperative and antagonistic interplay between PU.1 and GATA-2 that results in specification of alternate cell fates, mast cells, or macrophages. We demonstrate that PU.1 antagonizes GATA-2 expression during macrophage differentiation, but functions cooperatively with GATA-2 to specify the mast cell fate. This leads us to propose a developmental model in which reciprocal antagonism between PU.1 and GATA family factors specifies macrophage or erythrocyte fates, whereas cooperative interaction between PU.1 and GATA-2 is essential for mast cell development.

Results

PU.1 Regulates the Proliferation and Differentiation of Mast Cell Progenitors In Vivo

During mouse embryogenesis, mast cells can be observed in subcutaneous tissues by day 15.5 of development (Kitamura et al., 1979). Since the targeted *PU.1* null mutation results in late embryonic lethality (Scott et al., 1994), we examined dermal tissue from day 16.5 *PU.1*−/− fetuses for the presence of mast cells. These cells can be visualized by metachromatic staining of their basophilic granules with toluidine blue. Similar numbers of mast cells were observed in the dermis of *PU.1* +/+ and +/− fetuses (Figure 1A, left panel, and

data not shown). Strikingly, no mast cells were detectable in tissue from *PU.1*−/− fetuses (Figure 1A, right panel). Thus, the *PU.1* gene is essential for mast cell development, in vivo.

The defect in mast cell development caused by the *PU.1* mutation may be due to either a failure to generate mast cell progenitors or a failure of such progenitors to undergo differentiation. To address this issue, we performed methylcellulose colony-forming assays in the presence of IL-3, SCF, or their combination. *PU.1*+/− fetal liver progenitors formed three distinct types of myeloid colonies in IL-3 (mast, granulocyte/macrophage, and mixed). As reported previously, SCF synergized with IL-3 to induce mast cell colony formation (Lantz and Huff, 1995). The *PU.1* mutation caused a severe reduction (approximately 100-fold) in the number of IL-3-responsive progenitors (see supplemental data at http://www.immunity.com/cgi/content/full/17/5/665/DC1). Furthermore, the few colonies that did form consisted of immature cells lacking morphological characteristics of mast cells, macrophages, and neutrophils (data not shown). These results suggest that PU.1 is important for the survival/proliferation of mast cell progenitors in vivo and is also required for their differentiation.

Establishment of IL-3-Dependent *PU.1*−/− Progenitor Cell Lines

To analyze PU.1 function in mast cell differentiation, IL-3-dependent cell lines were established from *PU.1*+/− and −/− hematopoietic progenitors. Previous studies have shown that IL-3 promotes the generation of immature mast cells from bone marrow or fetal liver derived hematopoietic progenitors. Hematopoietic progenitors (Lin−) were isolated from day 14.5 *PU.1*+/− and −/− fetal liver and cultured in IL-3 (see Experimental Procedures). Immature mast cells were detectable in *PU.1*+/− cultures after 21 days and predominated after 60 days (Figure 1B, left panel). These cells possessed an abundant cytoplasm containing varied numbers of basophilic granules characteristic of immature mast cells. They exhibited small nuclei in relation to their cytoplasm. The combination of SCF and IL-3 induced further differentiation of the *PU.1*+/− cells, as evidenced by increased accumulation of secretory granules (Figure 1B). Even though IL-3-responsive progenitors were severely reduced by the *PU.1* mutation, they expanded readily in IL-3, facilitating the establishment of *PU.1*−/− cell lines. The mutant cells showed characteristics of myeloid progenitors (Figure 1B, right panel). They exhibited large lobular nuclei and reduced cytoplasm with many vacuoles. Some of these vacuoles contained material which stained faintly with toluidine blue. Unlike their *PU.1*+/− counterparts, *PU.1*−/− cells did not differentiate further in response to IL-3 and SCF (Figure 1B). Western analysis confirmed the absence of PU.1 protein in the *PU.1*−/− cells (see below). These mutant cells appeared to be blocked for mast cell differentiation at an early developmental stage.

PU.1−/− Cells Are Defective for Secretory Granule Biogenesis and FcεRI Expression

To analyze the molecular defect in secretory granule biogenesis caused by the *PU.1* mutation, we examined the expression of various mast cell-specific proteases, which are normally found in the granules of immature mast cells in vitro. Immunocytochemistry revealed that unlike their *PU.1*+/− counterparts, *PU.1*−/− cells failed to express mMCP-5 protein (Figure 2A). This defect in mMCP-5 expression was due to a failure to express the gene (Figure 2B). Transcripts encoding other mast cell proteases, mMCP-2 and mMCP-4, were also absent in *PU.1*−/− cells (Figure 2B and data not shown, respectively). Transcripts for mMC-CPA, a protease expressed early in mast cells, were detectable in *PU.1*−/− cells, albeit at approximately 20-fold reduced levels. Thus, *PU.1*−/− cells are unable to accumulate secretory granules in part because of a failure to express genes encoding the protease components induced early in mast development.

We next determined whether loss of PU.1 affects the developmental induction of FcεRI expression, in addition to blocking granule biogenesis. Analysis of *PU.1*−/− cells revealed an absence of surface expression of FcεRI, compared to their heterozygote counterparts (Figure 2C). The block in surface expression of FcεRI was due to a failure to accumulate transcripts encoding the FcεRIα and β subunits (Figure 2D). Interestingly, FcεRIγ transcripts were detected at equal levels by RT-PCR in both *PU.1*+/− and −/− cell lines (data not shown). Thus, PU.1 is required for expression of genes encoding the α and β subunits of FcεRI as well as mast cell-specific proteases.

Retroviral Transduction of PU.1 into the Mutant Cells Induces Mast Cell and Macrophage Differentiation

To determine if the IL-3-dependent *PU.1*−/− cells are developmentally competent to undergo mast cell differentiation, we transduced them with a retroviral vector containing *PU.1* cDNA and the gene for enhanced green fluorescent protein (MSCV-EGFP-PU.1, see Experimental Procedures). To enrich for productively infected cells, we FACS sorted on the basis of GFP expression. Two weeks after FACS sorting, mutant cells transduced with MSCV-EGFP-PU.1, but not the control vector (MSCV-EGFP), gave rise to a subpopulation that expressed both GFP and FcεRI (Figure 3A). The MSCV-EGFP-PU.1-infected cultures contained morphologically distinguishable mast cells with some metachromatic cytoplasmic granules (Figure 3B, right panel). Granule accumulation in such cells was further stimulated by culturing in SCF and IL-3 (data not shown). Thus, *PU.1*−/− cells can be induced to differentiate into immature mast cells by reexpression of PU.1. It should be noted that a fraction of the GFP^+ cells expressing PU.1 differentiated into macrophages (Figure 3B, right panel). However, the macrophages did not proliferate in IL-3 and therefore mast cells were selectively expanded. In order to confirm that the IL-3-dependent *PU.1*−/− cells also had macrophage potential, we transduced them with PU.1 and cultured in M-CSF on S17 stromal cells instead of in IL-3. Under these conditions, the PU.1-transduced progenitors efficiently differentiated into macrophages but not mast cells (Figure 3C, right panel). Collectively, these results suggest that the *PU.1*−/− cells represent multipotential myeloid progenitors which can be induced to differentiate into mast cells or macrophages upon reexpression of PU.1.

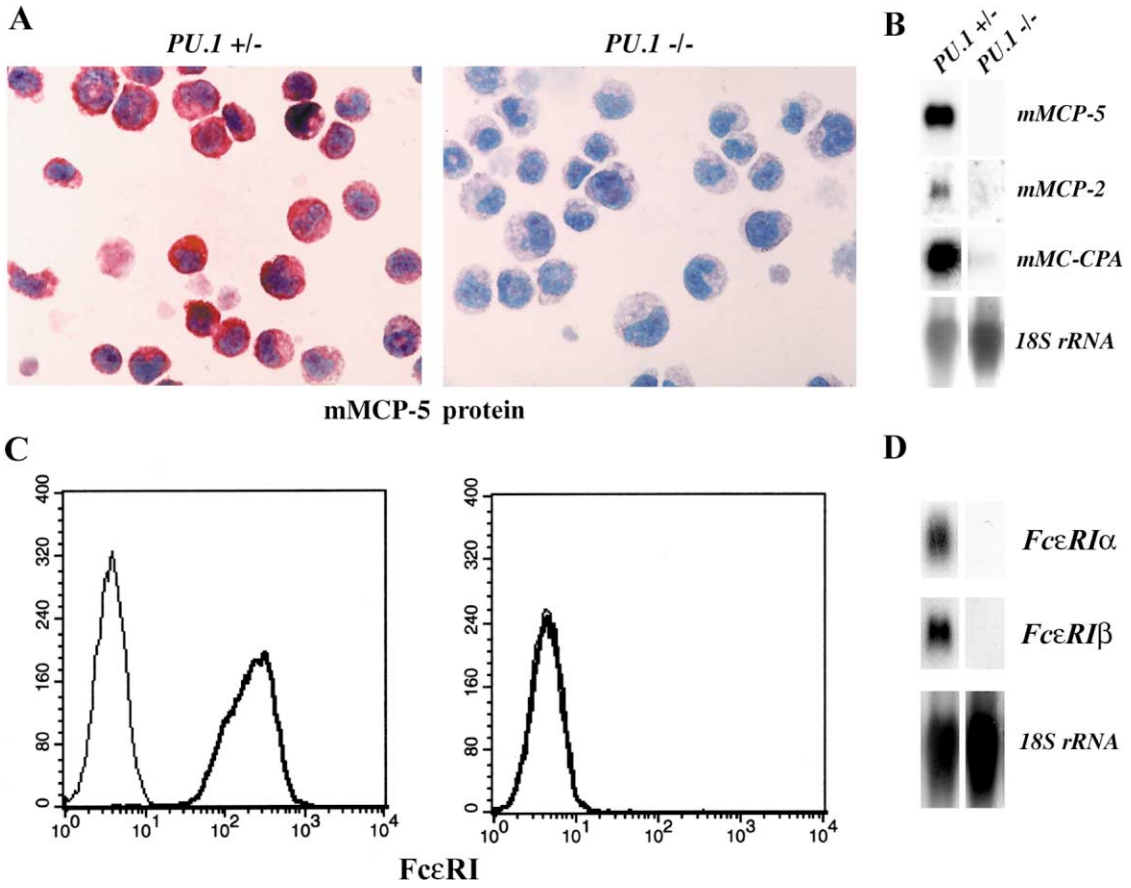

Figure 2. PU.1 Is Required for the Expression of Mast Cell Proteases and FcεRI

(A) Immunocytochemical staining of mMCP-5 in IL-3-dependent *PU.1*+/− and −/− cell lines. Red staining of cytoplasm indicates presence of mMCP-5 in *PU.1*+/− cells.

(B) Northern blot analysis of transcripts encoding mast cell-specific proteases mMCP-5, mMCP-2, and mMC-CPA.

(C) FACS analysis of IL-3-dependent *PU.1*+/− and *PU.1*−/− cells stained with IgE (bold line).

(D) Northern blot analysis of transcripts encoding the α and β subunits of the high-affinity IgE receptor, FcεRI.

Generation of *PU.1*−/− Cells Expressing Conditionally Activatable PU.1 Proteins

To examine how PU.1 regulates the specification of distinct myeloid cell fates (mast cell versus macrophage), an inducible version of the protein was generated by fusion to the ligand binding domain of the estrogen receptor (PUER, Figure 4A). The estrogen receptor domain is a variant (ERTM) that is preferentially regulated by tamoxifen (OHT). Two mutant PUER derivatives were also generated by fusing the transactivation (TA) and PEST regions of PU.1 (ΔC111) or the Ets DNA binding domain (ΔN160) to the ERTM segment. An IL-3-dependent *PU.1*−/− progenitor cell line was transduced with retroviral vectors expressing either the full-length PU.1 fusion protein or the mutant derivatives. Initially, pools of stably transduced cells were selected and then clones were obtained by limiting dilution. The clones used for in-depth analysis had the same properties as the pools from which they were derived. Expression of the individual PU.1 fusion proteins was examined by Western blotting (Figure 4B). The full-length fusion protein was expressed at higher levels compared with its ΔC111 and ΔN160 derivatives.

Developmental Potential of PUER-Transduced *PU.1*−/− Progenitors

In the absence of tamoxifen (OHT), the PUER protein displayed basal activity as it induced low-level Mac-1 expression on the surface of *PU.1*−/− progenitors (Figure 4C). The gene encoding the CD11b subunit of Mac-1 appears to be directly regulated by PU.1 since its promoter contains a functionally important binding site for PU.1 and its expression is PU.1 dependent (Chen et al., 1993; DeKoter et al., 1998). The basal activity of the PU.1 fusion protein did not, however, induce morphological differentiation (Figure 4D). When the PUER-expressing cells were cultured in 100 nM OHT, they differentiated into macrophages (Figure 4D) expressing high levels of Mac-1 (Figure 4C) and the macrophage colony stimulating factor receptor (data not shown). As shown previously, PU.1-induced macrophage differentiation required both the Ets DNA binding domain and the transactivation domain (Fisher et al., 1998). The cells expressing the mutant fusion proteins (ΔC111 or ΔN160) did not undergo differentiation upon tamoxifen treatment (data not shown). These control cell lines demonstrate that differentiation of PU.1−/− progenitors into

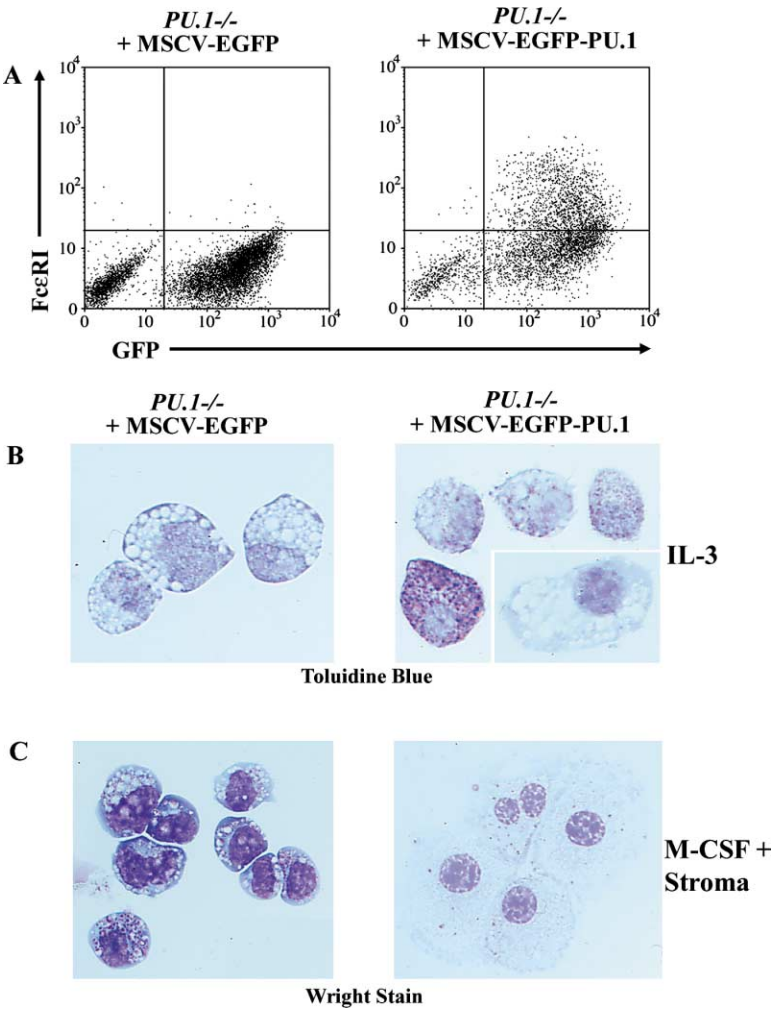

Figure 3. Expression of PU.1 in IL-3-Dependent Mutant Cells Induces Mast Cell and Macrophage Differentiation

(A) PU.1−/− cells were infected with either MSCV-EGFP or MSCV-EGFP-PU.1 retrovirus and cultured in complete medium with IL-3 for 2 days. Infected cells were enriched by FACS sorting for green fluorescence protein and analyzed for FcεRI expression after 14 days of culture in IL-3.

(B) Toluidine blue staining of PU.1−/− cells from the experiment described above (original magnification 1000×). Right panel shows immature mast cells as well as a macrophage.

(C) PU.1−/− cells after infection with either MSCV-EGFP or MSCV-EGFP-PU.1 retrovirus were cultured in M-CSF on S17 stromal cells for 14 days and analyzed by Wright staining.

macrophages is not simply due to tamoxifen treatment and/or expression of the ERTM protein segment. The activated PUER protein also inhibited IL-3-dependent proliferation of the *PU.1*−/− cells. When cultured in 100 nM OHT, the PUER cells failed to proliferate after completing one round of cell division (data not shown). It should be noted that 100 nM OHT treatment of the parental *PU.1*−/− cells neither affected their proliferation in IL-3 nor induced their differentiation. Furthermore, neither the ΔC111 nor the ΔN160 fusion proteins induced growth arrest upon OHT treatment. Thus, it is the induction of PU.1 activity by OHT that results in inhibition of IL-3-dependent proliferation of PU.1−/− progenitors and their differentiation into macrophages.

Surprisingly, activation of the PUER protein in *PU.1*−/− progenitors did not result in the generation of mast cells. As described above, the mutant progenitors, when infected with a retroviral vector which constitutively expresses PU.1, can give rise to both macrophages and mast cells. We therefore reasoned that expression of high levels of the PUER protein may have altered the developmental capacity of the cells, due to its basal activity. Indeed, the PUER fusion protein was expressed at considerably higher levels than PU.1 in the mutant cells after retroviral transduction (Figure 4E). We focused our analysis on GATA-2 since it is essential for mast cell but not macrophage development. Intriguingly, whereas the *PU.1*+/− and −/− cells expressed the GATA-2 protein, the PUER cells did not (Figure 4F). This suggested that PU.1 can downregulate GATA-2 expression, and such antagonistic crossregulation may result in a loss of mast cell developmental potential.

PU.1 Downregulates Expression of the *GATA-2* Gene during Macrophage Differentiation

The expression status of the *GATA-2* gene in the PUER cells was analyzed by RT-PCR (Figure 5A). As was the case for GATA-2 protein, the PUER cells did not express the *GATA-2* gene. Intriguingly, expression of the *GATA-2* gene was maintained in ΔN160 cells but downregulated in the ΔC111 cells (Figure 5A). As shown earlier, both mutant fusion proteins are expressed at equivalent levels and neither is capable of promoting myeloid differentiation. Downregulation of the *GATA-2* gene by PU.1 represents specific antagonistic crossregulation since PUER did not impair expression of other key hematopoietic transcription factor genes such as *AML1*, c-*Myb*, and *SCL/Tal-1* (Figure 5A). In these cells, the high levels of the PUER protein and its basal activity appear to be sufficient to downregulate *GATA-2*. The PUER protein

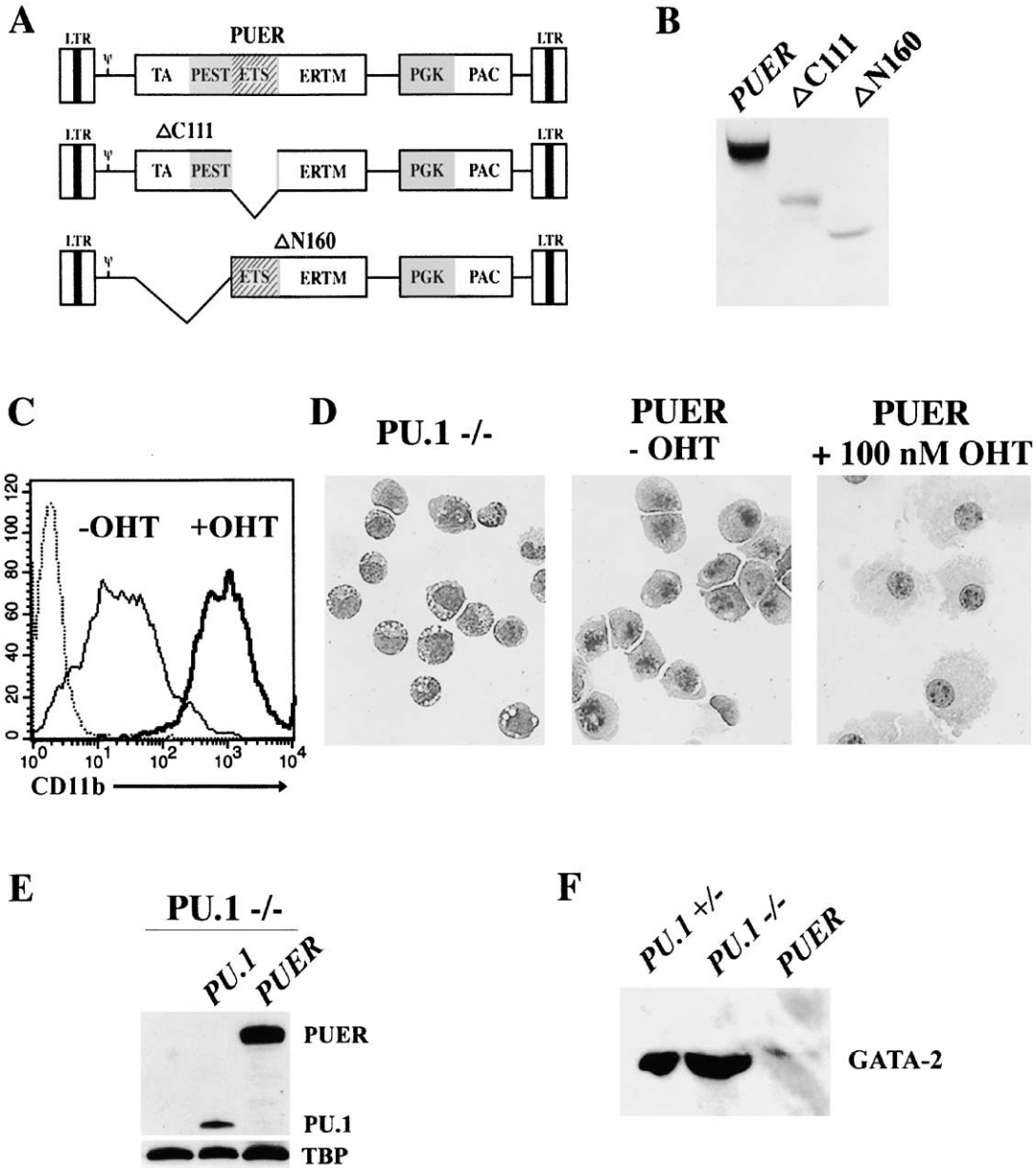

Figure 4. A Conditionally Activatable PU.1 Protein Induces Macrophage but Not Mast Cell Differentiation

(A) Schematic depicting the structures of MSCV-PAC retroviral vectors encoding various PU.1 derivatives fused to the tamoxifen responsive ligand binding domain of the estrogen receptor (ERTM).

(B) IL-3-dependent *PU.1*−/− cells were infected with the indicated vectors, and pools of stably tranduced cells were selected using puromycin. Expression levels of the three PUER protein derivatives were analyzed by Western blotting.

(C) FACS analysis of Mac-1 expression on a representative PUER clonal line isolated from the pool described in (B). Cells were cultured in the absence (thin line) or presence of 100 nM OHT (thick line) for 3 days before FACS analysis. Dashed line represents Mac-1 expression on the parental *PU.1*−/− progenitor cell line.

(D) Analysis of macrophage differentiation induced by the PUER protein using Wright staining (original magnification 1000×). Cells described in (C) were cultured in 100 nM OHT for 7 days prior to cytochemical analysis.

(E) Western analysis of PU.1 and PUER expression in PU.1−/− progenitor cells after retroviral transduction. PU.1 expression was analyzed after transduction with MSCV-EGFP-PU.1 and sorting for GFP^+ cells (see legend to Figure 3). PUER cells are described in (C).

(F) Analysis of GATA-2 expression by Western blotting in the indicated IL-3-dependent cell lines.

was also expressed in *PU.1*−/− cells using a GFP-based retroviral vector system. This enabled isolation of PUER-expressing cells (GFP^+) immediately after retroviral transduction. In these experiments, we observed OHT-inducible downregulation of the *GATA-2* gene (Figure 5B). Furthermore, a PUER derivative that carries mutations in the recognition helix of the ets DNA binding domain still downregulated the *GATA-2* gene (data not shown). Thus the PU.1-mediated downregulation of the *GATA-2* gene is neither dependent on the Ets DNA bind-

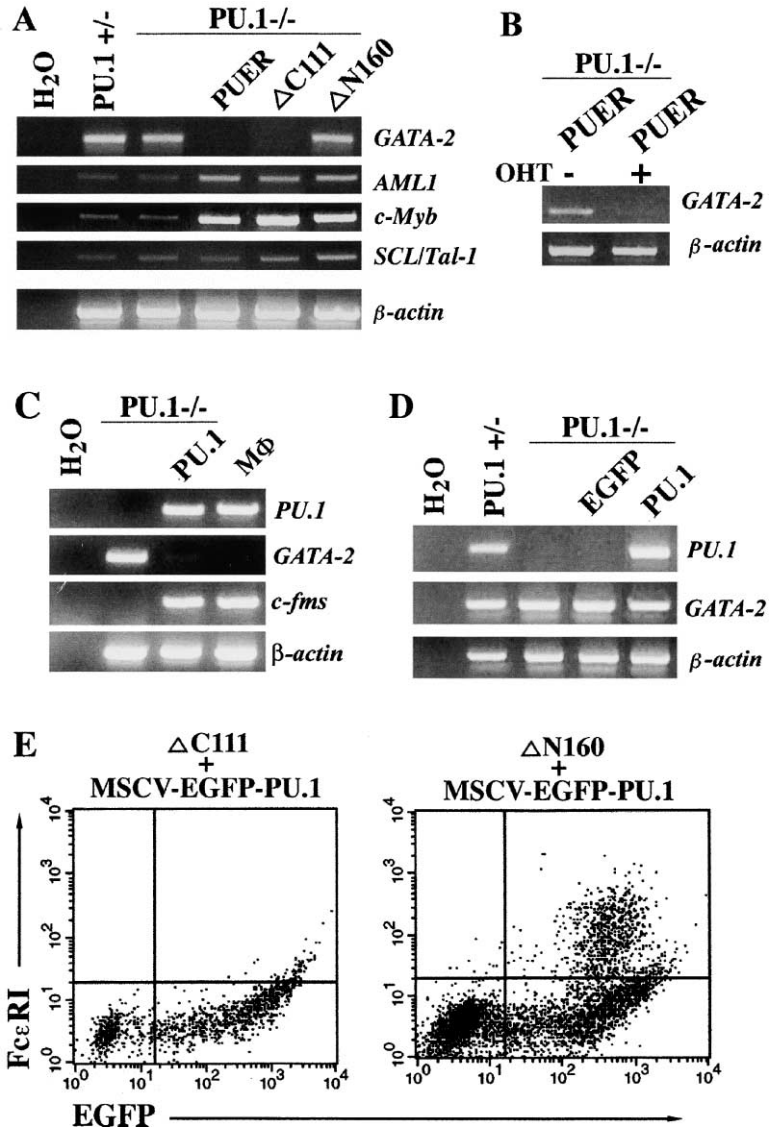

Figure 5. PU.1 Can Downregulate *GATA-2* Gene Expression Thereby Causing a Block to Mast Cell Development

(A) RT-PCR analysis of transcripts encoding various hematopoietic transcription factors in the indicated IL-3-dependent cell lines (see Figure 4B).

(B) RT-PCR analysis of GATA-2 expression in PU.1−/− progenitors after transduction of PUER. The mutant progenitor cells were transduced with an MSCV retroviral vector expressing PUER and GFP. Two days after infection, the cells were sorted for GFP expression and then cultured in the absence (−) or presence (+) of 100 nM OHT for 24 hr before isolating RNA.

(C) RT-PCR analysis of *GATA-2* expression in macrophages generated from wild-type progenitors or by transduction of PU.1 into *PU.1*−/− cells (see legend to Figure 3C).

(D) RT-PCR analysis of GATA-2 expression in mast lineage cells (PU.1+/−) and PU.1−/− progenitors induced to differentiate into mast cells by retroviral transduction of PU.1 (see legend to Figures 3A and 3B).

(E) FACS analysis of FcεRI and GFP expression in ΔC111 or ΔN160 cells after superinfection with MSCV-EGFP-PU.1 retrovirus. Virally transduced cells were propagated in IL-3 for 14 days prior to FACS analysis.

ing domain nor its DNA binding activity (Figure 5A and data not shown).

The above experiments suggested that PU.1 may physiologically downregulate the expression of the *GATA-2* gene during the differentiation of myeloid progenitors into macrophages. To examine this possibility, expression of the *GATA-2* gene was analyzed after inducing the differentiation of *PU.1*−/− progenitors into macrophages by retroviral transduction with PU.1 and culturing the cells with M-CSF and the stromal cell line S17 (see Figure 3C). *GATA-2* expression was downregulated in PU.1-rescued macrophages derived from *PU.1*−/− progenitors as well as in macrophages generated from wild-type hematopoietic progenitors (Figure 5C). Importantly, GATA-2 expression was sustained in PU.1−/− cells that were induced to become mast cells (see Figures 3A and 3B) after retroviral transduction of PU.1 (Figure 5D). These experiments demonstrate that PU.1 specifically downregulates the expression of the *GATA-2* gene during the differentiation of myeloid progenitors into macrophages but not mast cells.

Cooperative Interplay between PU.1 and GATA-2 in the Specification of Mast Cell Fate

As stated above, we hypothesized that PU.1 inhibition of *GATA-2* gene expression precludes mast cell differentiation and therefore restricts the developmental capacity of myeloid progenitors. This proposal is consistent with the sustained expression of the *GATA-2* gene during mast cell development but its downregulation during macrophage differentiation (Figures 5A and 5C).

We tested the hypothesis that PU.1 functions in combination with GATA-2 to specify the mast cell fate using two distinct experimental strategies. In the first approach, we took advantage of the ΔC111 and ΔN160 cells which differentially express GATA-2, but are incapable of differentiating into mast cells or macrophages because they lack intact PU.1. To test their mast lineage developmental potential, the ΔC111 and ΔN160 cells were transduced with the PU.1 retrovirus. Stably transduced cells were sorted on the basis of GFP expression and cultured in IL-3 for 2 weeks. As stated above, mast cells are selectively expanded under these conditions

since they continue to proliferate in IL-3 whereas macrophages do not. Expression of PU.1 in the ΔN160 cells, which retain *GATA-2* expression (Figure 5A), resulted in the generation of immature mast cells that expressed the high-affinity IgE receptor (FcεRI, Figure 5E) and accumulated secretory granules in their cytoplasm (data not shown). No mast cells were generated by expression of PU.1 in the ΔC111 cells which lack *GATA-2* expression. These results demonstrate that loss of *GATA-2* expression eliminates mast cell developmental potential.

A second test of our hypothesis entailed reexpression of GATA-2 in the PUER cells. To perform this test, we superinfected the PUER cells with retroviruses expressing either GFP or hGATA-2 along with GFP (Figure 6). Stably transduced pools of control (PUER+GFP) or GATA-2-expressing PUER (PUER+GATA-2) cells were then cultured in the absence or presence of 100 nM OHT. Like the parental PUER cells (Figure 4C), the control PUER+GFP cells generated macrophages upon induction with 100 nM OHT. They did not express FcεRI or accumulate secretory granules characteristic of mast cells (data not shown). In striking contrast, activation of PU.1 in the PUER+GATA-2 cells induced the expression of the mast cell marker FcεRI (Figure 6B, right panel). The generation of mast cells by the combined action of PU.1 and GATA-2 was documented by the following criteria: (1) induction of FcεRI expression (Figure 6B); (2) maintenance of c-kit expression on a subset of the cells after induction of PU.1 activity (data not shown); (3) accumulation of secretory granules in the cytoplasm (Figures 6C and 6D); and (4) sustained proliferation in IL-3. It should be noted that, in the presence of 100 nM OHT for 7 days, the PUER+GATA-2 cells generated mostly macrophages and relatively few morphologically distinct mast cells (Figure 6C). However, because immature mast cells retain the ability to proliferate in IL-3, they represented the predominant cell type after an additional 14 days of culture (Figure 6D). These experiments demonstrate that the induction of PU.1 activity in a multipotential hematopoietic progenitor, under conditions which sustain GATA-2 expression, enables the specification of the mast cell fate.

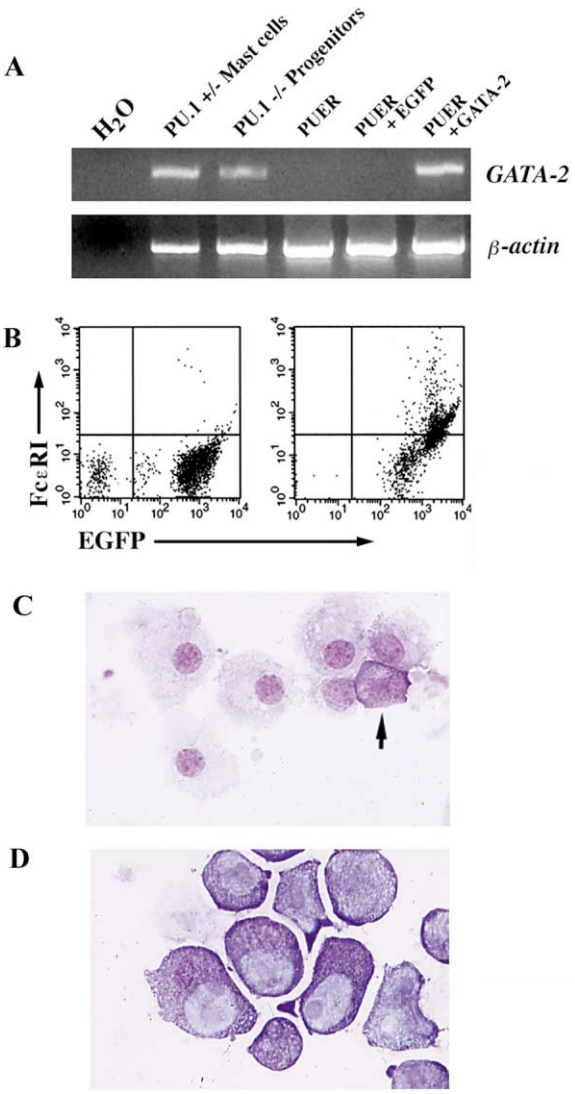

Figure 6. PU.1 Cooperates with GATA-2 to Specify the Mast Cell Fate

(A) RT-PCR analysis of *GATA-2* transcripts in the indicated cells. A PUER clone (see Figure 4C) was superinfected with either a control MSCV-EGFP retrovirus or a hGATA-2 encoding derivative to establish the PUER+EGFP and PUER+GATA-2 cell lines, respectively (see Experimental Procedures).

(B) FACS analysis of PUER+GATA-2 cells in the absence (left panel) or presence (right panel) of 100 nM OHT. Cells were cultured for 7 days with OHT prior to FACS analysis.

(C) Wright staining of the PUER+GATA-2 cells after 7 days of culture in OHT (original magnification 1000×). Arrow indicates granulated cell with immature mast cell morphology.

(D) Toluidine blue staining of PUER+GATA-2 cells after 21 days of culture in OHT.

Discussion

PU.1 is a hematopoietic transcription factor that is uniquely required for the development of macrophages and neutrophils, as well as B and T lymphocytes (Singh et al., 1999). Although PU.1 is expressed in mast cells and implicated in regulating mast cell-specific gene expression (Henkel and Brown, 1994), its developmental function in the mast cell lineage remained to be explored. Using a previously described targeted mutation of the *PU.1* gene (Scott et al., 1994), we demonstrate that PU.1 functions to regulate both the proliferation as well as differentiation of mast cell progenitors. PU.1 is required for early events in mast cell differentiation including granule biogenesis and expression of FcεRI. Furthermore, our results demonstrate that PU.1 functions in concert with GATA-2 to induce multipotential progenitors to differentiate into immature mast cells.

PU.1 Is Required for Proliferation as Well as Differentiation of Mast Cell Progenitors

Disruption of the *PU.1* gene results in the complete absence of embryonic dermal mast cells as well as a severe reduction in IL-3 and SCF responsive hematopoietic progenitors in the fetal liver that give rise to mast cells. We have previously shown that *PU.1*−/− hematopoietic progenitors can proliferate in vitro in response to the

multilineage cytokines IL-3, IL-6, and SCF, but are unresponsive to the myeloid-specific cytokines GM-CSF, G-CSF, and M-CSF (DeKoter et al., 1998). Here, we demonstrate that SCF promotes colony formation by *PU.1*−/− progenitors in the presence of IL-3 (see supplemental data at http://www.immunity.com/cgi/content/full/17/5/665/DC1). Furthermore, *PU.1*−/− progenitors expanded with IL-3 express c-kit albeit at lower levels than their heterozygous counterparts and can proliferate in response to SCF (data not shown). Therefore, the reduction in mast cell progenitors caused by the *PU.1* mutation is not simply due to an absence of c-kit expression or signaling.

The ability to culture *PU.1*−/− hematopoietic progenitors in IL-3 enabled a detailed analysis of the block to mast cell differentiation. *PU.1*−/− progenitors are defective in secretory granule biogenesis (Figure 1). The mutant cells contain small vesicles, but are largely devoid of granular material. The defect in granule biogenesis is in part due to the failure to express genes encoding granule components. In particular, genes encoding mast cell proteases mMCP-2, mMCP-4, and mMCP-5 are not expressed in the mutant cells (Figure 2). Expression of mMC-CPA, although detectable, is reduced approximately 20-fold. By comparison, the earliest defined mast cell precursors express transcripts for the proteases mMC-CPA, mMCP-2, and mMCP-4 and contain a few secretory granules (Rodewald et al., 1996). Culturing of such precursors in IL-3 and SCF promotes further differentiation accompanied by induction of FcεRIα transcripts and surface expression of FcεRI. *PU.1*−/− progenitors, cultured in IL-3 or the combination of IL-3 and SCF, neither express FcεRI on their surface nor transcripts encoding the FcεRIα and β subunits (Figure 2), consistent with an early block to mast cell differentiation. On the basis of this phenotypic comparison, *PU.1*−/− progenitors arrest before the mast cell precursor stage.

The block to mast cell development induced by the *PU.1* mutation appears to be at the level of a multipotential progenitor, i.e., prior to specification along the mast lineage. First, of the various mast cell markers examined, the IL-3-dependent *PU.1*−/− progenitors express detectable transcripts of only one, *mMC-CPA*. Second, the *PU.1*−/− progenitor cells transduced with *PU.1* cDNA give rise to mast cells as well as macrophages (Figure 3). Most importantly, a clonal line of *PU.1*−/− progenitors harboring a conditionally activatable PUER fusion protein generates macrophages and mast cells in the presence of GATA-2 (Figure 6). Thus the IL-3-dependent *PU.1*−/− cells represent multipotential myeloid progenitors and provide a unique system for analyzing distinct myeloid differentiation pathways.

PU.1 and GATA-2 Function in Concert to Regulate Early Events in Mast Cell Differentiation

The role of GATA-2 in mast cell development has been most directly examined by establishing long-term yolk sac cultures from *GATA-2*−/− embryos in IL-3 and SCF (Tsai and Orkin, 1997). Under these conditions, *GATA-2*−/− yolk sac cells generate macrophages but not mast cells. This in vitro system did not permit a detailed analysis of GATA-2 function in mast cell development, since *GATA-2*−/− hematopoietic progenitors readily differentiate into macrophages. Using a similar in vitro culture system (expansion of *PU.1*−/− fetal liver progenitors in IL-3), we have been able to analyze the role of PU.1 in mast cell differentiation. This is made possible by the fact that *PU.1*−/− progenitors unlike *GATA-2* −/− progenitors cannot differentiate into either macrophages or mast cells in the presence of IL-3. Using this system and gain-of-function experiments, we demonstrate that both PU.1 and GATA-2 are required for mast cell differentiation. The DNA binding as well as transactivation domains of PU.1 are essential for promoting mast cell differentiation (Figure 5). We suggest that PU.1 and GATA-2 may coregulate gene expression in mast cell precursors. Transcription of the mMC-CPA gene, an early marker of mast lineage cells, appears to require both PU.1 (Figure 2B) and a GATA factor (Zon et al., 1991). Similarly, transcription of the IL-4 gene in mast cells appears to be regulated by an enhancer which is PU.1- and GATA-factor dependent (Henkel and Brown, 1994). These observations suggest that mast cell gene expression may be driven by combinatorial action of PU.1 and a GATA factor on regulatory regions containing binding sites for both factors. GATA-2 rather than GATA-1 is most probably regulating early mast cell gene expression since the former is essential for development (Tsai and Orkin, 1997) and is expressed at higher levels in immature mast cells (Harigae et al., 1998). Importantly, these considerations imply that PU.1 and GATA-2 do not antagonize each other's transactivating functions in mast cells (see below).

Regulatory Interactions between PU.1 and GATA Factors in Erythroid/Myeloid Differentiation

Multipotential erythroid/myeloid progenitors appear to express high levels of GATA-2 and low levels of GATA-1 and PU.1 (Akashi et al., 2000). Differentiation of these progenitors into myeloid cells such as macrophages is preceded by the induction of PU.1 expression and the downregulation of GATA-1 and GATA-2 expression (Cheng et al., 1996) and Figure 5. In contrast, erythroid differentiation is accompanied by the induction of GATA-1, and a low level of PU.1 expression. Development of erythrocytic precursors is dependent on both GATA-1 and GATA-2 (Pevny et al., 1995; Tsai and Orkin, 1997) and occurs independent of PU.1 expression (Scott et al., 1994). Furthermore, ectopic GATA-1 expression in an avian myelomonocytic cell line can induce reprogramming into an erythroblast (Kulessa et al., 1995). In the avian system, GATA-1 can inhibit PU.1 expression (McNagny et al., 1998). Recently, ectopic expression of PU.1 in *Xenopus* embryos has been shown to inhibit erythropoiesis which is overcome by elevated expression of GATA-1 (Rekhtman et al., 1999). Thus, GATA-1 inhibition of PU.1 expression and/or function appears to represent a critical regulatory switch underlying erythroid differentiation (Figure 7). Intriguingly, induction of PU.1 activity in transformed avian multipotential progenitors promotes myeloid differentiation and down-regulation of GATA-1 expression (Nerlov and Graf, 1998). Thus, downregulation of GATA-1 by PU.1 appears to represent a reciprocal regulatory switch for promoting macrophage differentiation (Figure 7).

We demonstrate that both the native PU.1 protein as

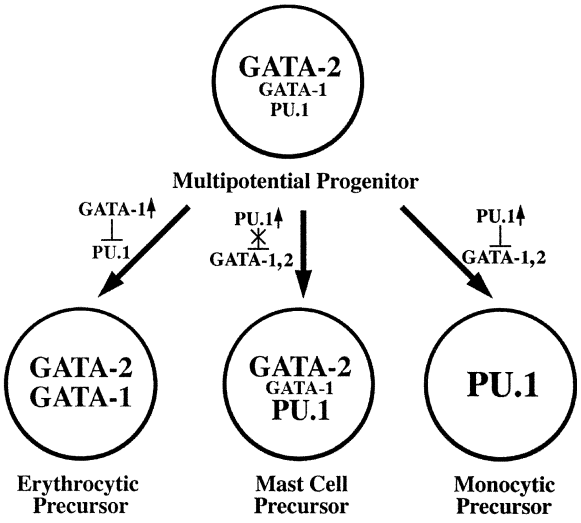

Figure 7. A Developmental Model for the Cooperative and Antagonistic Interplay between PU.1 and GATA Factors in the Specification of Hematopoietic Cell Fates

The model depicts the crossregulatory interactions among GATA-1, GATA-2, and PU.1, in the development of erythrocytes, macrophages, and mast cells from a common multipotential progenitor. In contrast to the antagonistic roles of GATA factors and PU.1 in macrophage and erythrocyte development, these regulators cooperate to enable specification of the mast cell lineage. Sizes of fonts for GATA-1,2 and PU.1 symbolize relative levels of expression and/or activity states.

well as its PUER derivative can downregulate GATA-2 gene expression during monocytic development (Figures 5B and 5C). We suggest a novel regulatory function of PU.1, i.e., downregulation of *GATA-2* gene expression in the absence of overt myeloid differentiation. We note that the IL-3-dependent *PU.1*−/− progenitors express low levels of *GATA-1* transcripts and that PU.1 as well as PUER also represses *GATA-1* gene activity (data not shown). We propose that the inhibition of *GATA-2* and *GATA-1* gene activity by PU.1 in a multipotential progenitor is crucial in restricting the developmental capacity of this cell (Figure 7). Such a cell can no longer give rise to erythrocytes, megakaryocytes, or mast cells since GATA-1 and/or GATA-2 (Tsai et al., 1994; Pevny et al., 1995) are required for the development of these lineages. It should be noted that PU.1-mediated downregulation of the GATA-2 gene is probably necessary for proper macrophage differentiation, as the conditional expression of GATA-2 in an ES cell system results in a block to macrophage development (Kitajima et al., 2002). Our experiments demonstrate that the antagonistic interplay between PU.1 and GATA-2 results in an alteration of the developmental capacity of a multipotential hematopoietic progenitor. We suggest that such interactions among hematopoietic regulatory genes in multipotential progenitors precede overt differentiation and represent molecular programming of specific developmental fates.

How does PU.1 repress the activity of the *GATA-2* gene? We note that repression of the *GATA-2* gene by PU.1 is independent of the ERTM segment used in our PUER fusion, as *GATA-2* is downregulated in macrophages generated by transduction of wild-type *PU.1* cDNA into the *PU.1*−/− cells (Figure 5C). Our results demonstrate that repression of the *GATA-2* gene by PU.1 does not require the ets DNA binding domain, thus ruling out direct binding of PU.1 to a regulatory sequence in the *GATA-2* gene. The amino-terminal segment of PU.1 which downregulates *GATA-2* gene expression contains transcriptional activation domains as well as the PEST region. Although the mechanism by which the amino-terminal segment of PU.1 regulates GATA-2 expression remains to be analyzed, an attractive possibility is suggested by the ability of this domain to interact with GATA-2 (Zhang et al., 1999). Such an interaction may impair GATA-2 function, which could result in GATA-2 gene downregulation, assuming an autoregulatory loop.

Although reciprocal inhibitory interactions among PU.1 and the GATA factors underlie the specification of macrophage versus erythroid cell fates, we show that the generation of mast cells is dependent on cooperative interplay between PU.1 and GATA-2 (Figure 7). In the mast lineage, PU.1 not only fails to repress *GATA-2* gene activity, but appears to coordinately function with GATA-2 in regulating mast cell-specific gene expression. How PU.1-mediated inhibition of *GATA-2* gene expression is circumvented in mast lineage cells is a key issue that requires further experimentation. It is possible that high levels of PU.1 expression or activity are required for inhibiting *GATA-2* gene expression. In this view, graded levels or activity of PU.1 could be used to specify mast versus macrophage cell fates. Differing levels of PU.1 have been shown to promote B lymphocyte versus macrophage development (DeKoter and Singh, 2000). Our results clearly establish that both antagonistic as well as cooperative interplay between hematopoietic transcription factors can be used to specify distinct cell fates. Such duality of regulatory interactions permits a small set of transcription factors, acting in combinatorial capacities, to specify multiple lineages from a common progenitor.

Experimental Procedures

Analysis of Embryonic Mast Cells and Their Progenitors

Day 16.5 *PU.1* +/+, +/−, and −/− embryos were fixed in Bouin's solution, dehydrated, and embedded in paraffin. 7 μm sections were stained with acidified toluidine blue (Sigma). Metachromatic-staining mast cells were counted along 1 cm regions of dorsal subcutaneous tissue (Kitamura et al., 1979).

Methylcellulose colony-forming assays were performed on day 14.5 embryonic fetal liver cells as described by DeKoter et al. (1998) using 10 ng/ml IL-3 and/or 100 ng/ml CSF. Colonies were stained with Wright or toluidine blue to examine composition of cell types.

Establishment and Analysis of IL-3-Dependent *PU.1*+/− and −/− Cell Lines

Day 14.5 *PU.1*+/− and −/− fetal liver progenitors were isolated and depleted of lineage-positive cells ($CD4^+$, $CD5^+$, $CD8a^+$, $CD3\epsilon^+$, $Gr\text{-}1^+$, $Ter119^+$, and $B220^+$). Lineage-depleted (Lin^-) hematopoietic progenitors were stimulated for 4 days in complete Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 1 U/ml penicillin-streptomycin, 100 ng/ml mSCF (Biosource), 10 ng/ml mIL-3 (Biosource), and 10 ng/ml mIL-6 (R&D Systems). Cell lines were then established by maintaining cytokine-stimulated hematopoietic progenitors in complete IMDM medium containing only 5 ng/ml IL-3 at cell densities between 10^5 and 10^6 cells/ml. After 60 days in culture,

PU.1+/− cell lines were >95% positive for FcεRI. The *PU.1*−/− cell lines did not express FcεRI nor the lineage-specific markers Ter119, Mac-1, B220, or Thy-1; however, they did express low levels of Gr-1.

Western blots were performed as described by DeKoter et al. (1998) using an affinity-purified anti-PU.1 antibody (Scott et al., 1997), rabbit anti-TBP (Santa Cruz #sc-273), or anti-murine GATA-2 antibody (kind gift of Dr. J. D. Engel).

Morphological, RNA, and FACS Analysis

For morphological analysis of *PU.1*+/− and −/− cell lines, 10^5 washed cells were cytospun onto glass slides, fixed for 30 s in methanol, and stained with Wright stain or toluidine blue. Cells were photographed using a Zeiss Axiovert microscope. Granule immunohistochemistry was carried out with anti-mMCP-5 Ig (McNeil et al., 1992), as previously described (Friend et al., 1996). RNA was isolated using RNAzol (Tel-Test, Inc.) and analyzed by Northern blotting or RT-PCR.

Flow cytometric analysis was performed on cells stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies: Anti-mouse monoclonal antibodies (mAb) M1/70 (CD11b, Mac-1), 2B8 (c-*kit*, CD117), and IgE-3 (IgE anti-DNP) (Pharmingen). Mouse IgE-3 was FITC-labeled or biotinylated using standard methods. Biotinylated IgE-3 was visualized with streptavidin-PE (Pharmingen). Fc Block (Pharmingen) was used to confirm specific binding of IgE-biotin to FcεRI on mast lineage cells.

Construction of Retroviral Vectors

Fusion of PU.1 to the ERTM segment was accomplished by amplifying an HA-tagged full-length *PU.1* cDNA lacking a stop codon. PU.1 deletion mutants containing either the transactivation domain and PEST region (ΔC111) or the Ets DNA binding domain (ΔN160) were fused to the ERTM in a similar manner. PUER fusion cDNAs were sequenced and in vitro translated to confirm their expression prior to subcloning into MSCV-PAC retroviral vector (kind gift of Dr. R. Hawley). MSCV-EGFP and MSCV-EGFP-PU.1 retroviruses have been described previously (DeKoter et al., 1998). Generation of the MSCV-EGFP vector expressing GATA-2 was accomplished by cloning an hGATA-2 cDNA fragment (kind gift of Dr. S. Orkin) into the MSCV-EGFP vector.

Retroviral Production and Transduction of *PU.1*−/− Progenitors

ϕNX-Eco retroviral packaging cells (Kinsella and Nolan, 1996) were used to generate retroviral supernatants which were further concentrated using a stirred-cell ultrafiltration apparatus (Amicon).

IL-3-dependent *PU.1*−/− cell lines were transduced by resuspension in cell-free retroviral supernatants for 3 hr in the presence of 50 μg/ml polybrene. Transduced cells were cultured for 2 days in complete medium containing 5 ng/ml IL-3 and then enriched for GFP expression by one round of cell sorting using a FACSCalibur cell sorter (Becton-Dickinson). After 14 days of culture in IL-3, cells were analyzed for expression of FcεRI and mast cell granule accumulation. For efficient macrophage differentiation, IL-3-dependent PU.1−/− cells were transduced with the PU.1 retroviral vector and plated on S17 stromal cells with M-CSF as described in DeKoter and Singh (2000). With PUER retroviruses, which contain the puromycin resistance gene, infected *PU.1*−/− cells were cultured in IL-3 containing media for 2 days prior to selection in 1 μg/ml puromycin. Clones were generated from pools of puromycin-resistant cells, after plating, by limiting dilution. Introduction of hGATA-2 into clonal *PU.1*−/− progenitors expressing PUER was accomplished by superinfection with MSCV-EGFP-hGATA-2 retrovirus, followed by two rounds of FACS sorting for expression of GFP, allowing 1 week between sorts for expansion of cells. Except where noted, retroviral transduced cells were cultured in media containing IL-3.

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